

(Progress Report  
NSG-643

(The Bacteriology of "Clean Rooms")

by

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During the six-month period covered by this report (4-1-65 to 9-30-65) progress can be summarized in four different areas as follows:

I. Identification of spore formers and other heat resistant micro-organisms actually recovered from fallout strips in conventional aero-space "clean room" facilities.

II. Experimental determination of dry heat resistance of the specifically identified spore formers, with particular reference to the 135° C. for 24 hours cycle.

III. Experiments related to human contact contamination of various representative materials and simulated components.

IV. Progress toward the design and construction of a small laminar downflow facility for future evaluation.

## I. IDENTIFICATION OF HEAT RESISTANT ORGANISMS ISOLATED FROM INDUSTRIAL CLEAN ROOMS

An attempt has been made to identify heat resistant organisms isolated from 1" x 2" fallout strips located in Industrial Clean Rooms A and D at Minneapolis Honeywell and Univac respectively. Evaluations of these strips were summarized in the previous progress report.

Colonies were picked from the aerobic plates to tubes of brain heart infusion broth and incubated at 32° C. After 48 hours, tubes showing growth were streaked on trypticase soy agar plates, incubated for 24 hours and picked to TAM sporulation agar slants<sup>1)</sup>. Broth tubes showing no growth were held for one week before being discarded as negative.

Smears were made from the TAM slants after 48 hours incubation at 32° C. to check for purity, Gram reaction and sporulation. If no sporulation was apparent, slants were re-incubated and checks made at 48 hour intervals for spores. After eight days incubation if no sporulation had occurred, organisms were transferred to "G" medium<sup>2)</sup>, and the above procedure repeated. A total of three organisms have resisted all attempts to induce sporulation.

After sporulation was observed, isolates were identified using procedures and media set forth by Smith, Gordon, and Clark<sup>3)</sup>. All identification was based on microscopic morphology and biochemical reactions. No agglutinations or bacteriophages have been employed.

Many organisms did not fit precisely into the key of Smith, et al., nor into any of the four additional species described in "Bergey's Manual of Determinative Bacteriology", 7th edition. These organisms have been placed arbitrarily in the species they most closely resemble as described by the above authors.

All initial isolations were carried out on trypticase soy agar at 32° C.; therefore, *Bacillus stearothermophilus*, which requires an incubation temperature of 60° C., and *Bacillus pasteurii* which needs urea or alkaline conditions for growth, would not have been isolated had they been present on the strips.

The results, to date, of these identifications are summarized in Table I. It is not surprising that virtually all isolates appear to be very common environmental spore forming bacteria.

TABLE I

Species Identification of Spore Formers Isolated From Stainless Steel Strips in Conventional Aerospace "Clean Rooms"

<u>Species</u>	<u>No. of Isolates</u>
<i>B. subtilis</i>	33
<i>B. pumilus</i>	28
<i>B. megaterium</i>	6
<i>B. cereus</i>	6
<i>B. pantothenicus</i>	1
<i>B. circulans</i>	1
<i>B. laterosporus</i>	1
Actinomycetales*	<u>1</u>
Total	77

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\*This organism identified only to order, not to genus and species.

## II. DETERMINATION OF THERMAL DEATH TIMES OF SPECIFIC "CLEAN ROOM" SPORE FORMERS

This procedure was carried out on selected isolates identified as described in Section I.

The procedure can be outlined as follows:

1. The organism is identified as a spore former (put into Group 1, 2, or 3 on the basis of Smith's system of classification)<sup>3)</sup>.

2. The organism is streaked on trypticase soy agar and incubated for 20 hrs @ 32° C.

3. A check is made with Gram's stain for purity, if culture is not pure a restreak is made on trypticase soy agar and incubated for 20 hrs @ 32° C.; a Gram's stain is prepared to check for purity. This procedure is continued until a pure culture is obtained.

4. The single pure isolate from trypticase soy agar is streaked on "G" agar<sup>2)</sup> and incubated for 48 hrs @ 32° C. A spore stain is prepared to check for degree of sporulation and purity. If 70% sporulation is not achieved, then the culture is allowed to grow for an additional 72 hrs. (This is to assure sufficient concentration for preparation of test aliquot. All cultures analyzed to date have reached this degree of sporulation).

5. Two to five isolates from "G" agar are inoculated into a flask containing 75 mls of "G" broth; the flask is heat shocked for 15 minutes @ 80° C., placed on a mechanical shaker and incubated for 72 hrs @ 32° C. A spore stain is made. If 70% sporulation has not been achieved, incubate an additional 48 hrs and check for per cent of sporulation. If not at least 70%, a new culture is started.

6. The temperature of the culture is reduced to 5° C. for 15 minutes,

then 20 mls of the culture is put into a sterile screw cap centrifuge tube and centrifuged at 2,000 rpms for 60 minutes @ 1° C. The supernatant is poured off and replaced with 20 mls of sterile distilled water.

7. The 20 ml suspension is diluted to obtain a concentration of approximately 100-1000 spores per 0.1 ml of aliquot. A  $10^{-5}$  dilution of the reconstituted spore suspension generally yields this concentration.

8. A determination of concentration of spores in each aliquot (of the  $10^{-5}$  dilution) is checked in the following manner; 0.01, 0.05, and 0.1 ml volumes of the spore suspension are plated in 100 mls of tryptone glucose yeast extract agar, incubated 72 hrs @ 32° C. and enumerated by means of a Bactronic Colony Counter.

9. 0.1 ml of dilute spore suspension is deposited on a clean, sterile 1" x 2" stainless steel strip contained in a sterile Petri plate; the inoculum is spread out with a wire, and with the Petri plate covers ajar, allowed to dry in a hood equipped with an absolute filter.

10. Four groups (10 strips/group) are prepared in this manner. A positive control is run with each culture tested.

11. At 135° C. one group of ten strips is challenged to 15 minutes, one group to 30 minutes, one group to 60 minutes and one group to 24 hrs of dry heat in a hot air convection oven equipped with a constant recording thermometer.

12. After heat treatment each strip is placed individually into 50 mls of tryptone glucose yeast extract broth and allowed to incubate for 14 days at 32° C. All transfers are made in the hood with absolute filter.

13. The data are reported as a ratio of the number of positive strips (demonstrating growth in TGY broth) to the total number of strips

tested in each series. The positive control strips must demonstrate growth in TGY broth.

14. Selected tubes of organisms from bottles demonstrating growth, as indicated by turbidity, are verified as similar to the original inoculum by biochemical, morphological and other routine microbiological studies.

Results of these determinations are summarized in Table II. It can be seen that there was not a single positive strip following 24 hours at 135° C. and very few strips had survivors after just one hour at that temperature.

TABLE II

Dry Heat Resistance of Spore Forming Bacteria Isolated from Conventional Aerospace "Clean Rooms"

Species			Ratio of Positive Strips to Total Strips Following Heat Shock at 135° C.			
	No. of Isolates Heat Shocked	Mean Spores Per Strip	15 min.	30 min.	60 min.	24 hours
Bacillus subtilus	14	437	114/140	22/140	2/140	0/140
Bacillus megaterium	2	265	1/15	0/15	0/15	0/15
Bacillus pumilus	15	774	105/146	18/144	0/145	0/145
Bacillus cereus	4	468	4/40	0/40	0/40	0/40
Actinomycetales *	1	3217	2/10	0/10	0/10	0/10
TOTAL	36	-	226/351	40/349	2/350	0/350

\*This organism identified only to order, not to genus and species.

### III. EXPERIMENTS RELATED TO HUMAN CONTACT CONTAMINATION OF VARIOUS REPRESENTATIVE MATERIALS AND SIMULATED COMPONENTS

It is generally recognized that human beings are a principal source of microbial contaminants in clean room areas. Therefore, information concerned with contamination levels transferred by workers in assembly processes, as well as the efficacy of control procedures, are obviously of interest to the aerospace program.

Hand contact appears to be one of the important mechanisms for transmission of microorganisms from the assembly line technician to his immediate surroundings. Because they come into intimate contact with the nose, mouth, body surfaces, and clothing, as well as dust, soil, and many contaminated objects, the worker's hands are especially vulnerable to the accumulation of heavy contamination.

For this reason, a series of experiments now in progress at our laboratories is being directed toward obtaining additional data about the extent, persistence and control of microbial contamination transferred by the hands of persons. Although not yet completed, these investigations include the following:

#### Study A

An analysis of the degree of contamination deposited on different component materials by contact and manipulation with hands and fingers.

#### Study B

A determination of die-off rates of natural heterogeneous microbial populations deposited on surfaces of various materials as a result of human contamination.

#### Study C

Studies concerned with the effectiveness of different control measures for reducing microbial contact contamination in simulated small component assembly processes.

Methods:

Tests of contamination deposited by handling were conducted on 1" x 2" strips of the following seven materials: (a) stainless steel of 28 gauge and with No. 5 or 6 finish, (b) 1/32" aluminum, (c) 1/32" epoxy glass laminate, (d) 1/16" copper covered epoxy laminate, (e) 2 oz. rolled copper plated with lead-tin solder, (f) 1/16" lucite, and (g) 1/32" teflon.

Simulated component assembly trials were done with 1/4" steel machine screws, washers, and nuts.

All materials were thoroughly cleansed by washing, in turn, with hot detergent solution, hot tap water, hot distilled water, isopropyl alcohol, and then ether rinsed and drained dry. Dry heat sterilization at 180° C. for 90 minutes was used to kill any remaining microorganisms (except for heat labile materials which were steam autoclaved at 110° C. for 30 minutes).

Levels of contamination resulting from human handling (Study "A") were studied using the 1" x 2" strips of different materials. All seven types of material were contaminated simultaneously. The sterile strips were handled according to a statistically designed, randomized protocol. A group of four laboratory workers (two males and two females) handled the strips in each trial. By having each strip handled by four persons, it was hoped that much of the expected individual variability could be counteracted. The individuals were positioned along a laboratory bench to simulate an assembly line. The first person of the group picked up the strip, manipulated it with fingers, turned it over once and then passed it on to the next individual in line. Strips were ultimately collected in sterile Petri dishes and analyzed. A total of four trials were conducted, each consisting of 25 strips of each material.

Aseptic microbiological techniques were scrupulously observed in the analyses. In randomized order, each strip was placed in 25 ml of warmed ( $48^{\circ}$  C.), M/15 phosphate buffer, pH 7, with 0.02% Tween "20", and shaken on a shaking machine for 5 minutes. Then strips were removed, embedded in Petri dishes containing 15 ml of molten TSA agar, and incubated at  $32^{\circ}$  C. for 72 hours after which a colony count was made.

The total 25 ml buffer eluate for each strip was poured into a 150 mm Petri dish containing a previously prepared 2 mm solidified TSA agar base. The shaker bottle was rinsed with approximately 25 ml of molten TSA agar which was added to the eluate plate. Lastly, an additional 100 ml of molten agar was added to the dish to complete the pour plate. All plating was done in special cabinets equipped with absolute filters. After 72 hours of aerobic incubation at  $32^{\circ}$  C., colonies were counted with a Bactronic Colony Counter.

Total counts of viable particles for each strip comprise the eluate counts plus the count of residual colonies detected on the embedded strip. Data are reported as viable colonies per strip. With each series, sterile strips were processed as controls to check on the techniques in plating.

Studies of microbial die-off rates (Study "B") were done on all seven strip materials, but each type of material was assayed in a separate trial. Groups of four persons contaminated these strips by handling procedures outlined in Study "A".

Sets of 50 strips were analyzed immediately after contamination. Similar sets of contaminated strips (50 per set for periods through 1 week, 25 per set for longer time intervals) were stored in sterile Petri dishes at  $72 \pm 4^{\circ}$  F. and  $60 \pm 10\%$  R.H. Counts of microorganisms surviving storage

were made after 1, 2, and 3 days and after 1, 2, 6, 12, and 20 weeks following contamination. Analysis of individual strips followed the protocol described in Study "A".

Effects of special contamination control procedures (Study "C") were evaluated by simulating component production operations through the assembly of previously sterilized machine screws, washer and nut combinations. Four operators, two male and two female, each assembled 20 units which were collected in sterile Petri dishes labelled for each person. Benches where assembly was done were previously cleansed with 70% ethanol.

After all "components" were completed, groups of five units from each individual's assemblies were placed in bottles of buffer solution and analyzed by the method described in Study "A". With this technique comparative studies were made of the following:

1. Open laboratory assembly with:
  - a. no hand care
  - b. two minute Ivory soap wash
  - c. two minute Phisohex wash
2. Assembly in a special cabinet purged by an absolute filtered air stream. Units were assembled with hand care similar to open laboratory combinations and an additional experiment using a two minute Phisohex wash plus sterile gloves.

#### Results:

All data reported in this study refer only to the aerobic, mesophilic, heterotrophic microorganisms. No attempt was made to enumerate other physiological forms. Data in Table III indicate the differences in

average numbers of microorganisms deposited on component materials when handled by groups of individuals.

These results indicate that observed mean concentrations of microbial deposition varied from one group to the next, apparently reflecting the influence of large individual variability. Furthermore, the mean levels of deposition also varied with the type of material tested.

With the exception of solder strips, the mean levels of contamination deposited by Group A were consistently higher than those of Group B. Data for Groups C and D indicate that contamination from these groups was of intermediate levels. Under the conditions of generally more intense microbial deposition from Group A, the greatest accumulated mean contamination occurred on lucite and epoxy laminate, with approximately 63 and 33 viable particles per strip, respectively. Scrutiny of combined means for all groups provides evidence which suggests that greater numbers of viable particles are retained on non-metallic materials than on metallic materials under similar conditions of contamination.

The experimental results obtained from studies of microbial survival on the seven materials demonstrate interesting differences. The mean concentrations of organisms initially deposited on these materials varied widely as may be seen in Table IV. Mean values of 240 viable particles were detected on stainless steel, while a mean level of only 17.7 viable particles occurred on aluminum. Presumably these variations resulted from differences in cleanliness of hands, or in shedding from hands, of the persons participating in these studies. Since these materials were handled by different groups of individuals on different days, such variation in concentrations of initial contamination might be expected.

Although this experiment is still in progress, the information collected to date suggests certain trends in microbial survival rates on the materials studied. Highest die-off rates were observed on stainless steel, where mean counts dropped from 239 viable particles to less than one, over a period of 20 weeks storage. Thus, it appears that simple storage effected a 99% reduction in the mean microbial plate count for the steel strips. The aluminum and solder plated strips yielded mean concentrations indicating a 90% reduction from the initial contamination levels after 12 weeks storage.

Lowest die-off rates were observed on copper strips and on the non-metallic materials (epoxy laminate, lucite and teflon). Although yielding lowered mean counts following storage, these materials still retained mean levels of 10 to 25 viable particles per strip after 12 weeks storage. Another point of great potential importance is the fact that preliminary determinations of types of microorganisms surviving after 12 and 20 weeks on all materials revealed a very high proportion of potential sporeforming species.

Data from the studies of hand care and special barriers to contamination revealed that certain procedures were effective for reducing the transfer of microorganisms to component materials (see Table V). Washing with ordinary soap yielded erratic results, including in one instance a four-fold increase in counts from the nut and screw assemblies.

Hexachlorophene soap scrubs appeared to be moderately effective in reducing transfer of contamination. The greatest contamination control was obtained with a combination of Phisohex scrub plus the use of sterile disposable gloves. Use of this procedure reduced mean levels of contamination

to less than one organism per assembled unit. These data indicate that even modest precautions in hand care combined with the use of effective barriers such as sterile gloves can reduce microbial contamination considerably below the levels attained under conditions prevalent under ordinary, uncontrolled circumstances. A variety of additional experiments are planned to further investigate these phenomena. Among these are assembly trials with larger and more complex units, the use of sterile tools and special dress requirements, and a series of studies in a laminar downflow room which is now under construction.

TABLE III  
Microbial Contamination Detected on  
Component Materials After Handling by Groups of Four Persons

Material	Mean Microbial Plate Count* Per 1 X 2 Inch Strip				
	Group A	Group B	Group C	Group D	Material Mean**
A. Metallics Stainless Steel Copper Aluminum Solder	14.6 16.9 17.1 10.2	7.4 3.3 3.1 18.9	2.2 3.8 6.3 2.4	8.4 6.3 10.9 3.8	8.2 7.6 9.4 8.8
All Metallics	-	-	-	-	8.5
B. Non-Metallics Epoxy Laminates Lucite Teflon	33.0 63.4 17.0	9.4 10.8 6.5	16.7 25.9 18.4	18.8 17.2 16.2	19.5 29.3 14.7
All Non-Metallics	-	-	-	-	21.1
Group Mean	24.6	8.5	10.8	11.6	

\*Represents mean count for 25 strips of each material per group.

\*\*Represents mean count averaged over all groups, a total of 100 strips per material.

Survival After Storage of Microorganisms on  
Aerospace Component Materials Contaminated Through Handling

TABLE IV

Storage Time (In Days)	Mean Microbial Plate Counts* Per 1 x 2 inch Strip						
	Stainless Steel	Copper	Aluminum	Soldered Copper	Epoxy Laminate	Teflon	Lucite
0	239.9	101.4	17.7	25.6	20.1	59.8	55.5
1	253.2	86.5	11.9	6.3	34.9	14.9	39.5
2	140.6	39.6	3.4	7.6	19.2	12.2	30.0
3	28.8	98.2	4.9	8.0	13.6	15.4	27.0
7	36.4	63.7	3.0	26.5	15.4	17.1	35.5
14	5.6	38.4	10.9	4.4	14.5	12.4	54.0
42	1.1	71.6	2.6	3.6	30.8	13.4	26.0
84	1.0	26.9	1.1	2.2	13.6	10.2	-
140	0.9	-	-	-	-	-	-

\*Represents mean counts of 50 strips for each time interval through one week and 20-25 strips for subsequent time intervals.

TABLE V  
Levels of Microbial Contamination Detected During  
Simulated Component Assembly\* Trials Under Different Control Methods

Microbial Colony Counts - TSA Medium				
Method	Per Five Assemblies			Per Single Assembly
	Mean	Median	Range	Mean
<u>Open Laboratory</u>				
No Hand Care	611.3	432.0	20-1904	122.6
Two Min. Ivory Soap Wash	66.5	31.5	11-280	13.3
Two Min. Phisoex Wash	6.6	2.0	0-37	1.3
<u>Specialaire Hoods</u>				
No Hand Care	77.1	57.0	12-288	15.4
Two Min. Ivory Soap Wash	299.3	46.5	0-3044	59.9
Two Min. Phisoex Wash	20.8	15.5	5-51	4.2
Two Min. Phisoex Wash Plus Sterile Gloves	0.9	0.0	0-10	0.2

\*Nut-washer-machine screw assemblies.

#### IV. LAMINAR DOWNFLOW FACILITY FOR FUTURE EVALUATION

Considerable effort was also expended in planning and analyzing for construction of a prefabricated laminar downflow room for carrying out future experiments in contamination control during simulated assembly procedures. The room will be small (8' x 10') with a 4' x 4' entryway. The room has been delivered and it is hoped that it will be constructed and in operation in the very near future.

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